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hepatocytes were able to recover from a 1-h, but not a 12-h exposure. Cell damage, as assessed by release of lactate dehydrogenase, lagged behind inhibition of protein synthesis. Only at a T-2 concentration of 1.0 µg/ml for 12-h followed by a 12-h recovery period, was release of lactate dehydrogenase significantly elevated over control values. Under the same parameters, protein synthesis was inhibited by 94%. The ultrastructural appearance of the cell membrane, nucleus, lysosomes, peroxisomes, and smooth endoplasmic reticulum remained unchanged. The two organelles which appeared altered by T-2 exposure were the rough endoplasmic reticulum and the mitochondria. Endoplasmic reticulum changes were limited to degranulation of attached ribosomes without dilation of the cisternae. Alterations were seen as early as 1-h at a T-2 dose of 0.01 µg/ml. After a dose of 1.0 µg/ml T-2 for 12-h, some mitochondria displayed one or more non-membrane bound translucent foci, some of which contained electron-dense cores.

Biochemical and Ultrastructural Effects of T-2 Toxin on Rat Hepatocytes in Vitro

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ABSTRACT

Cultured rat hepatocytes were treated with several doses of T-2 mycotoxin for either 1 or 12-h and with or without a 12-h recovery period. Inhibition of protein synthesis and release of lactate dehydrogenase were measured and correlated with ultrastructural changes, as assessed by transmission electron microscopy. Results indicated that, at a dose of 0.01 µg/ml, protein synthesis was inhibited within 1-h but recovered to near control levels with or without the continual presence of toxin. At the higher toxin dose (1.0 µg/ml), the hepatocytes were able to recover from a 1-h, but not a 12-h exposure. Cell damage, as assessed by release of lactate dehydrogenase, lagged behind inhibition of protein synthesis. Only at a T-2 concentration of 1.0 µg/ml for 12-h followed by a 12-h recovery period, was release of lactate dehydrogenase significantly elevated over control values. Under the same parameters, protein synthesis was inhibited by 94%. The ultrastructural appearance of the cell membrane, nucleus, lysosomes, peroxisomes, and smooth endoplasmic reticulum remained unchanged. The two organelles which appeared altered by T-2 exposure were the rough endoplasmic reticulum and the mitochondria. Endoplasmic reticulum changes were limited to degranulation of attached ribosomes without dilation of the cisternae. Alterations were seen as early as 1-h at a T-2 dose of 0.01 µg/ml. After a dose of 1.0 µg/ml T-2 for 12-h, some mitochondria displayed one or more non-membrane bound translucent foci, some of which contained electron-dense cores.

INTRODUCTION

T-2 toxin (3α-hydroxy-4β, 15-diacetoxy-8α (3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene) is a small molecular weight non-protein toxin produced by the Fusarium species (13), and is a very potent inhibitor of eucaryotic protein synthesis (6). In vitro studies have demonstrated that T-2 toxin is rapidly metabolized in isolated perfused rat livers (J.D. Pace and M. Watts, Fed. Proc. **42**:1809, 1983), and by cultured rat hepatocytes (J. O'Brien et al., Fed. Proc. **44**, 1038, 1985). In vitro effects of the mycotoxin, fusarenon, on liver cells have been examined, but few data are available on the biochemical and ultrastructural effects of T-2 on cultured hepatocytes.

The aim of the current study was to assess the ultrastructural effects of T-2 toxin on cultured hepatocytes and correlate this with release of lactate dehydrogenase and inhibition of protein synthesis. Since hepatocytes contain predominantly membrane-bound ribosomes, we were also interested in comparing the effects of T-2 to similar data from our previous studies with Chinese hamster ovary (CHO) and African green monkey kidney (VERO) cells, which contain predominantly non-membrane-bound ribosomes (L.R. Trusal, Cell Biochem. Func., in press).

MATERIALS AND METHODS

Hepatocyte cultures

Primary cultures of rat hepatocytes were prepared by collagenase perfusion (2) of livers of 125-225-g fed male Fisher Dunning 344 rats. Prior to surgery, animals were anesthetized with 100 mg/kg Vetalar (Parke-Davis, Morris Plains, NJ) and 12 mg/kg Rompun (Cutter Laboratories, Inc., Shawnee, KA). The final dispersed hepatocyte preparation was suspended in modified Williams E Medium (Flow Laboratories, Inc., McLean, VA) (1) at a concentration of $1x10^6$ cells/ml.

Toxin

T-2 toxin (Calbiochem-Behring, San Diego, CA) was maintained at a stock concentration of 5 mg/ml in ethanol. Just prior to use, toxin was diluted to the desired concentration (i.e. 0.01 or 1.0 µg/ml) in tissue culture medium.

Inhibition of protein synthesis assay

Hepatocytes used to study inhibition of protein synthesis were inoculated into 6-well dishes at a density of $1x10^6$ cells/ml. Following a 1-h attachment period, medium and unattached cells were aspirated and 1.0 ml of fresh medium with or without toxin was added. Protein synthesis was measured by modification of the method of Schwarze etal. (10). At the indicated times, toxin was removed, and media containing [^{3}H] valine (New England Nuclear, Boston, MA) at a specific activity of 10 µCi/ml was added. After 60 min, media was removed, phosphate buffered saline (PBS) was added, and cells were scraped. The cell suspension was centrifuged, and the pellet was resuspended in fresh PBS, followed by the addition of 0.2 N perchloric acid (3x, 0°C).

The final pellet was solubilized overnight in 0.3 N KOH (37°C), and a neutralized aliquot was assayed for radioactivity in a liquid scintillation counter (Mark III scintillation counter, Searle Analytic, Inc., Des Plaines, IL). Each data point represented triplicate wells and was presented as percentage (%) of incorporated radioactivity compared to a non-toxin control (% control).

Lactate dehydrogenase assay

Cell viability was measured by release of lactate dehydrogenase (LDH). The method involves determining the LDH activity in culture medium and comparing this to cells lysed with Triton X-100 (5). The LDH assay was performed on a COBAS BIO (Roche Analytical Diagnostic Systems, Nutley, NJ) (4).

Morphologic examination

Cultures used to study ultrastructure were seeded into Leighton tubes (Costar, Cambridge, MA) at a density of $1x10^6$ cells/tube with Williams E medium. Each tube contained a plastic (polymethylpentene) coverslip onto which the cells readily attached. Coverslips were processed for transmission electron microscopy (TEM) as previously described (11). Briefly, coverslips were rinsed, fixed in 2.5% phosphate-buffered glutaraldehyde, postfixed in 1% buffered osmium tetroxide, and dehydrated in ethanol. Coverslips were then embedded in Embedd 812 (Electron Microscopy Science, Fort Washington, PA) and polymerized for 3 days (56°C). Finally, ultrathin sections were cut and stained with uranyl acetate and lead citrate prior to viewing.

RESULTS

Release of LDH into the medium indicated that T-2 toxin has little effect on cell viability. Exposure of cells to 1.0 µg/ml T-2 toxin for 12-h resulted in no significant increase in LDH release, but after a 12-h recovery period, a slight signiffcant increase was noted (data not shown).

T-2-induced inhibition of protein synthesis in hepatocytes by T-2 toxin is summarized in Table 1. Exposure of cells to T-2 (0.01 µg/ml) for 1-h resulted in a 75% decrease in protein synthesis. Protein synthesis recovered tc near control levels after a 12-h recovery period. Interestingly, protein synthesis recovered to 87% of controls in cells continually exposed to T-2 (0.01 µg/ml) over 12-h. At the higher T-2 dose (1.0 µg/ml), protein synthesis was totally inhibited by 1-h. These cells only recovered if the toxin was removed for 12-h. This was in contrast to the longer T-2 exposure (12-h) where no recovery of protein synthesis capability occurred, even after the 12-h of recovery.

When examined by TEM, control hepatocytes contained numerous mitochondria, peroxisomes, golgi, transport vesicles, and rough- and smooth-endoplasmic reticulum (R-ER, S-ER) (Fig. 1A,B,C). The R-ER was often peripherally localized, but was also randomly scattered throughout the cytoplasm or in close association with mitochondria. Numerous ribosomes studed the endoplasmic reticulum membranes (Fig. 1C). Smooth-endoplasmic reticulum was found almost exclusively near the cell periphery in small or large patches. Mitochondria presented generally dense matrices with matrix granules (Fig. 1B). The cristae tended to be thin structures with some possessing enlarged tubular tips (Fig. 1B). The nuclear morphology was also typical with a light rim of marginated chromatin, nuclear pores, prominent

nucleoli, and a double lamellar membrane (Fig. 1A). Glycogen deposits were rarely seen in any of the cells.

When cultured hepatocytes were exposed to 0.01 µg/ml of T-2 toxin for 1 or 12-h, many cells contained regions of R-ER degranulated of attached ribosomes (Fig. 2). In other cells, this was not evident and R-ER appeared unaltered. Mitochondria still appeared with a dense matrix, matrix granules, and thin cristae with tubular tips (Fig. 2). The nucleus, golgi apparatus, lysosomes, and peroxisomes appeared unaltered. At the higher dose of T-2 (i.e., 1.0 µg/ml) for 1 or 12-h, endoplasmic reticulum morphology was very similar. Areas containing parallel rows of R-ER were largely devoid of attached ribosomes (Fig. 3).

An unusual finding in some cells was the occurrence of mitochondria containing one or more translucent foci (Fig. 4A) in hepatocytes exposed to 1.0 µg/ml T-2 for 12-h. The foci numbered from 1 to 5 and were non-membrane bound. A limited number of foci also contained electron-dense cores (Fig. 4B). Mitochondria containing foci occurred in abundance in some cells and not at all in other cells. Mitochondria with foci were observed in two separate experiments in a limited number of cells but not in controls. In mitochondria containing foci, there was no increased matrical density, compartmental swelling, or disruption of the outer membrane.

When hepatocytes were allowed a recovery period following the high dose toxin exposure, protein synthesis recovered after a 1-h exposure but not after 12-h. This was paralleled by the TEM observations of the endoplasmic reticulum. Many of the cells exposed to 1.0 µg/ml T-2 for 1-h with a 12-h recovery period once again contained attached ribosomes (Fig. 5), while the majority of cells exposed for 12-h followed by a recovery period did not.

DISCUSSION

Assessment of cell viability by measurement of LDH release demonstrated that, under the experimental parameters, hepatocytes were not killed by T-2 toxin. This test is a standard biochemical marker of cellular membrane integrity and correlates well with both trypan blue exclusion (1) and hydrolysis of fluorescein diacetate (9). This finding is consistent with the ability of other cultured cells to maintain their membrane integrity following exposure to T-2. The plasma membranes of both Chinese hamster ovary (CHO) and African green monkey kidney (VERO) cells were shown by TEM not to undergo lysis following exposure to T-2 toxin (1.0 µg/ml, 12-h), although CHO cells did exhibit formation of surface blebs whereas VERO cells did not. (L.R. Trusal, Cell Biochem. Func., in press). We did not observe T-2-induced blebs in hepatocytes exposed to T-2.

The occurrence of degranulation of R-ER in T-2 treated hepatocytes is consistent with our observations of both CHO and VERO cells (L.R. Trusal, Cell Biochem. Func., in press) and a published report of human fibroblasts (8). In CHO and VERO cells, T-2 toxin induced total polysomal breakdown and degranulation of the limited amount of R-ER. In hepatocytes, which contain few polysomes, but do have extensive R-ER, degranulation of R-ER is consistent with inhibition of protein synthesis caused by the toxin (Table 1). Investigators have reported that other trichothecene mycotoxins (i.e., fusarenon, nivalenol) cause polysome breakdown in in vitro systems (14). They speculate that the susceptibility of a cell to trichothecenes is mainly dependent on the number of free polysomes in the cytoplasm, since differentiated cells with few free polysomes, such as hepatocytes, are not affected by fusarenon and nivalenol (14). This is in contrast to our studies with T-2 toxin, where we

demonstrated that T-2 caused dissociation of hepatic ribosomes from the endoplasmic reticulum. It is possible that such observations are related to in vivo (14) rather than in vitro studies reported here. It is difficult to determine to what particular toxin concentration or metabolite a specific cell type is exposed, in situ, in the whole animal. In addition, other factors, such as toxin binding, transport, differential clearance, metabolism, and molecular structure may be involved. It is also possible that, unlike nivalenol, the greater equal molar potency of T-2 disrupts both cytoplasmic polysomes and membrane-bound ribosomes.

While others have described T-2 induced irregularities in the nuclear envelope and chromatin margination patterns in human fibroblasts (7), we did not observe such changes in hepatocytes. Differences may again be related to different cell types, toxin concentrations, or time of exposure.

In a previous study, we noted mitochondrial changes in CHO and VERO cells exposed to T-2 toxin (L.R. Trusal, Cell Biochem. Func., in press) which included mitochondria with condensed configurations and cristae alterations. These observations were attributed to alterations in the mitochondria electron transport system (8,12) and inhibition of liver mitochondrial protein synthesis, known to be affected by T-2 exposure (J.G. Pace et al., Fed. Proc. 44, 1802, 1985). We saw no such ultrastructural changes in hepatic mitochondria exposed to identical doses of T-2. A plausible explanation lies in the greater ability of hepatocytes, compared to CHO and VERO cells, to metabolize T-2 toxin. Both CHO and VERO cells possess the ability for limited metabolism of T-2 toxin. After CHO and VERO cells undergo a 4-h exposure to 1.0 µg/ml T-2 toxin 50 to 67% of the counts remain as T-2 (L.R. Trusal and S.R. Watiwat, Fed. Proc. 43:578, 1984). On the other hand, hepatocytes converted 87% of T-2 to metabolites within 10 min. (Dr. Judith Pace,

manuscript in preparation). The ability of cultured hepatocytes to rapidly detoxify T-2 toxin to less toxic intermediates, such as T-2 triol and T-2 tetraol, may be responsible for the recovery of hepatocyte cultures illustrated in Table 1 (J.C. O'Brien et al., Fed. Proc. **44**, 1038, 1985). Longer exposures at higher doses (i.e. 1.0 µg/ml for 12-h) evidently overwhelm the hepatocytes' ability to detoxify the T-2 toxin. In addition, protein synthesis is irreversibly inhibited (Table 1).

It was at this higher T-2 dose for 12-h, that we observed a mitochondrial lesion not seen in CHO and VERO cells: the presence of mitochondria containing spherical foci (Figs. 4A,B). A similar, but not identical, pathogenesis has been described in porcine myocytes with acute monensin toxicosis (15), and skeletal muscle fibers with parasitic infection (3). The authors describe spherical lucent matrical foci in myocyte and skeletal muscle mitochondria, but do not speculate on reasons for the lesions. The lesions we observed occurred in two different experiments, but was prevalent in a limited number of cells exposed to 1.0 µg/ml T-2 for 12-h. One would speculate that more cells would be affected if there was a direct causual relationship between the occurrence of the lesions and toxin exposure. It is possible that the lesion occurs only after higher T-2 doses for longer time periods (i.e. $\geq$12-h), since we did not observe it except at a T-2 dose of 1.0 µg/ml. Support for this conclusion comes from the observation that the lesion was most prevalent after a 12-h exposure with or without a 12-h recovery period. The data (Table 1) establish that, at this dose and time point, protein synthesis is inhibited 85 to 95% in the total cell population, and hepatocytes are unable to recover from this exposure. It is possible that this lesion is specifically related to inhibition of mitochondrial protein synthesis in some cells of the total cell population. It is also possible the focal coagulation of proteins within

the mitochondria has occurred. We did not observe this specific lesion in CHO and VERO cells of our previous study. Additional studies are necessary to assess the true significance of mitochondria-toxin interactions and what role they play in the biological activity of the toxin on the cellular level.

In summary, in the present study, the hepatic organelles whose ultrastructure appeared altered by T-2 toxin were the endoplasmic reticulum and mitochondria. Endoplasmic reticulum changes were limited to degranulation of attached ribosomes without dilation of the cisternae. Some mitochondria contained round translucent foci of which some contained dense cores. Toxin exposure did not appear to affect the overall mitochondrial matrix density, the presence of matrix granules, the cristae shape, or the outer mitochondrial membrane integrity.

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LITERATURE CITED

1. **Casini, A., M. Gierli, R.J. Hyland, A. Serroni, D. Gilfor and J.L. Farber.** 1982. Mechanisms of cell injury in the killing of cultured hepatocytes by bromobenzene. J. Biol. Chem. **257**:6721-6728.

2. **Clark, D.G., R. Rognstad, and J. Katz.** 1974. Lipogenesis in rat hepatocytes. J. Biol. Chem. **249**:2028-2036.

3. **Despommier, D.** 1975. Adaptive changes in muscle fibers infected with Trichibella spiralis. Amer. J. Pathol. **78**:477-496.

4. **Gay, R.J., R.B. McComb, and G.H. Bowers, Jr.** 1968. Optimum reaction conditions for human lactate dehydrogenase isoenzymes as they affect total lactate dehydrogenase activity. Clin. Chem. **14**:740-753.

5. **Harman, A.W., and L.J. Fischer.** 1982. Alloxan toxicity in isolated rat hepatocytes and protection by sugars. Biochem. Pharmacol. **31**:3731-3736.

6. **McLaughlin, C., M. Vaughan, I.M. Campbell, C.M. Wei, M.E. Stafford, and B.S. Hansen.** 1974. Inhibition of protein synthesis by T-2. Biochem. Biophys. Res. Commun. **57**:834-844.

7. **Oldham, J.W., L.E. Allred, G.E. Milo, O. Kindig and C.C. Capen.** 1980. The toxicological evaluation of mycotoxins T-2 and T-2 tetraol using normal human fibroblasts in vitro. Toxicol. Appl. Pharmacol. **52**:159-168.

8. **Pace, J.G. 1983.** Effect of mycotoxin on rat liver mitochondria electron transport system. Toxicon **21**:675-680.

9. **Schanne, F.A., A.B. Kane, E.E. Young, and J.L. Farber.** 1979. Calcium dependence of toxic cell death: a final common pathway. Science **206**:700-702.

10. **Schwarze, P.E., A.E. Solheim, and P.O. Seglen.** 1982. Amino acid and energy requirements for rat hepatocytes in primary culture. In Vitro **18**:43-54.

11. **Trusal, L.R., C.J. Baker, and A.W. Guzman.** 1979. Transmission and scanning electron microscopy of cell monolayers grown on polymethylpentene coverslips. Stain Technol. **54**:77-83.

12. **Trump, B.F., and A.U. Arstila,** 1975. Cellular reaction to injury, p. 9-96. In M.F. LaVie and R.B. Hill (eds.), Principles of pathobiology, 2nd ed. Oxford Univ. Press, New York.

13. **Ueno, Y.** 1977. Trichothecenes: overview address, p. 189-207. In J.V. Rodricks, C.W. Hesseltine, and M.A. Mehlman (eds.), Mycotoxins in human and animal health. Pathotox, Park Forest South, IL.

14. **Ueno, Y.** 1983. Trichothecenes-chemical, biological and toxicological aspects, p. 147-162. In Y. Ueno (ed.), Developments in food science, vol. 4. Elsevier, New York.

15. **Van Vleet, J.F.** 1984. Ultrastructural alterations in the atrial myocardium. Amer. J. Pathol. **114**:367-379.

TABLE 1

Effects of T-2 mycotoxin on protein synthesis in hepatocytes

Exposure Time / Recovery Period		Percent Protein Synthesis[a]	
		0.01[b]	1.0[b]
1-h		25 +/- 0.41	0 +/- 0.63
1-h	12-h	87 +/- 2.20	87 +/- 3.00
12-h		87 +/- 2.90	14 +/- 0.46
12-h	12-h	100 +/- 8.60	6 +/- 1.90

[a] = Values are expressed as percentage of control protein synthesis +/- S.E.
[b] = Toxin dose is in µg/ml.

FIGURE LEGENDS

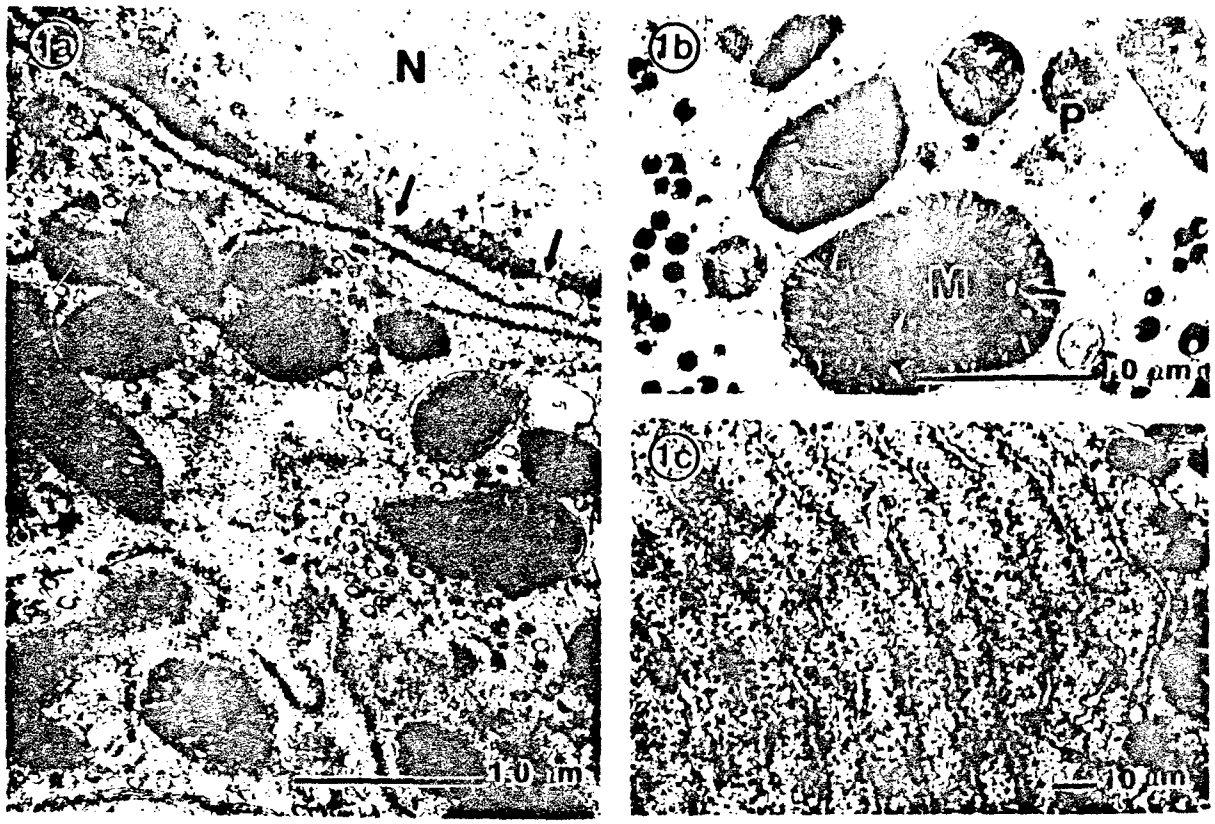
Fig. 1. - Electron micrographs of control hepatocytes. (A) Note intact nucleus with marginated chromatin and nuclear pores (arrows). (B) Mitochondria (M) contain dense matrixes, matrix granules, and some cristae with enlarged tubular tips (arrows). (C) Note intact R-ER with attached ribosomes.

Fig. 2. - Electron micrograph of an hepatocyte treated with 0.01 µg/ml of T-2 toxin for 1-h. Note generalized degranulation of R-ER (short arrows). Mitochondria are similar to controls with some cristae containing enlarged tubular tips (long arrows) and the presence of numerous microperoxisomes (MP).

Fig. 3. - Electron micrograph of an hepatocyte treated with 1.0 µg/ml T-2 toxin for 12-h. Note the similarity of organelle appearance to Fig. 2. The R-ER still retains its degranulated appearance (arrows).

Fig. 4. - Electron micrographs of hepatocytes treated with 1.0 µg/ml T-2 for 12-h. (A) Many mitochondria contain translucent foci (arrows). (B) A single mitochondria contains foci with electron-dense cores (arrows).

Fig. 5. - Electron micrograph of an hepatocyte treated with 1.0 µg/ml T-2 for 1-h followed by a 12-h recovery period in the absence of toxin. Note the attached ribosomes (arrows) on the endoplasmic reticulum and the lack of other ultrastructural changes.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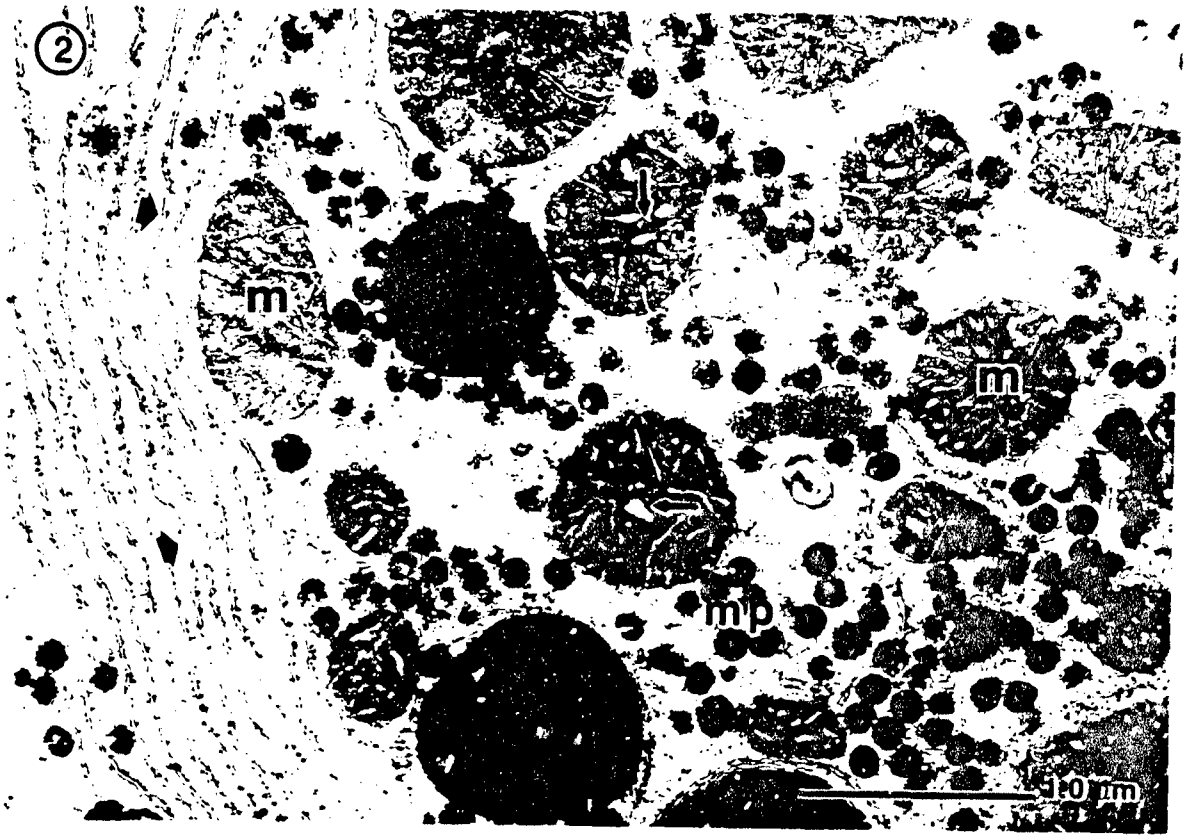
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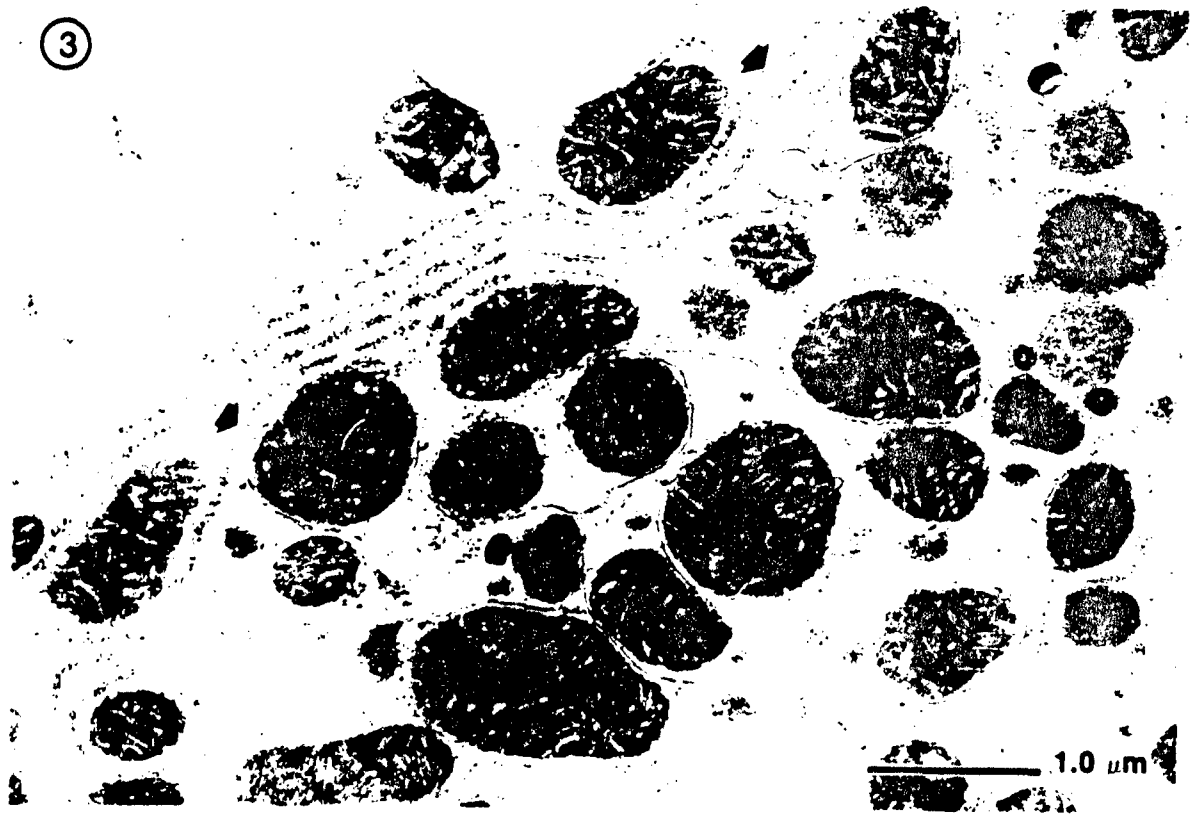
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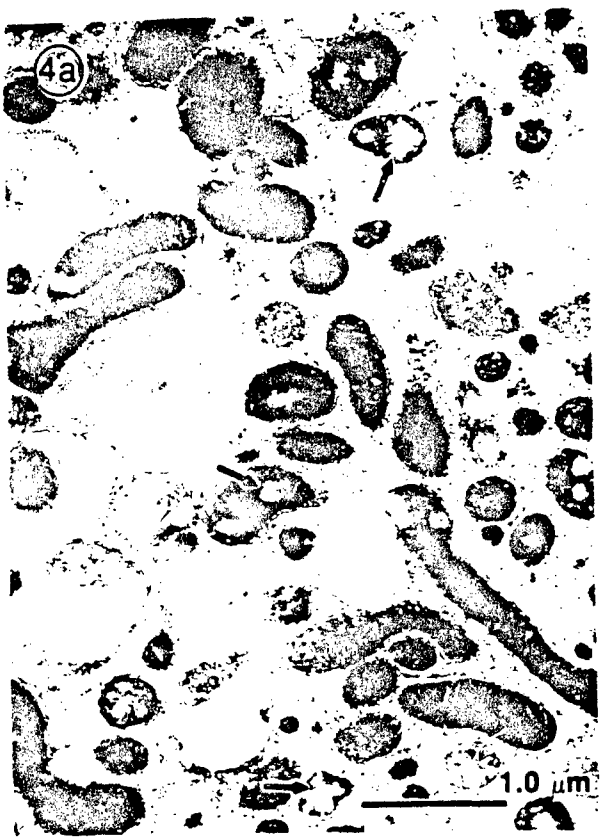
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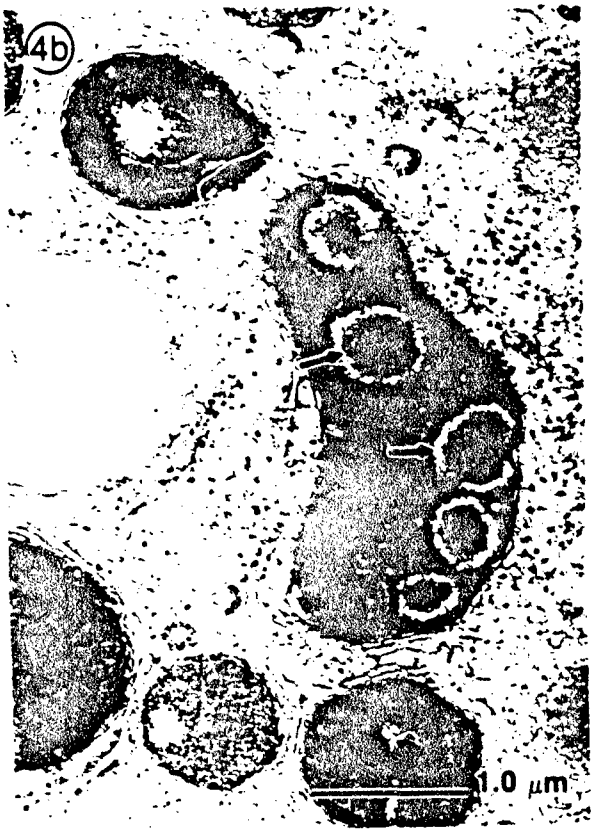
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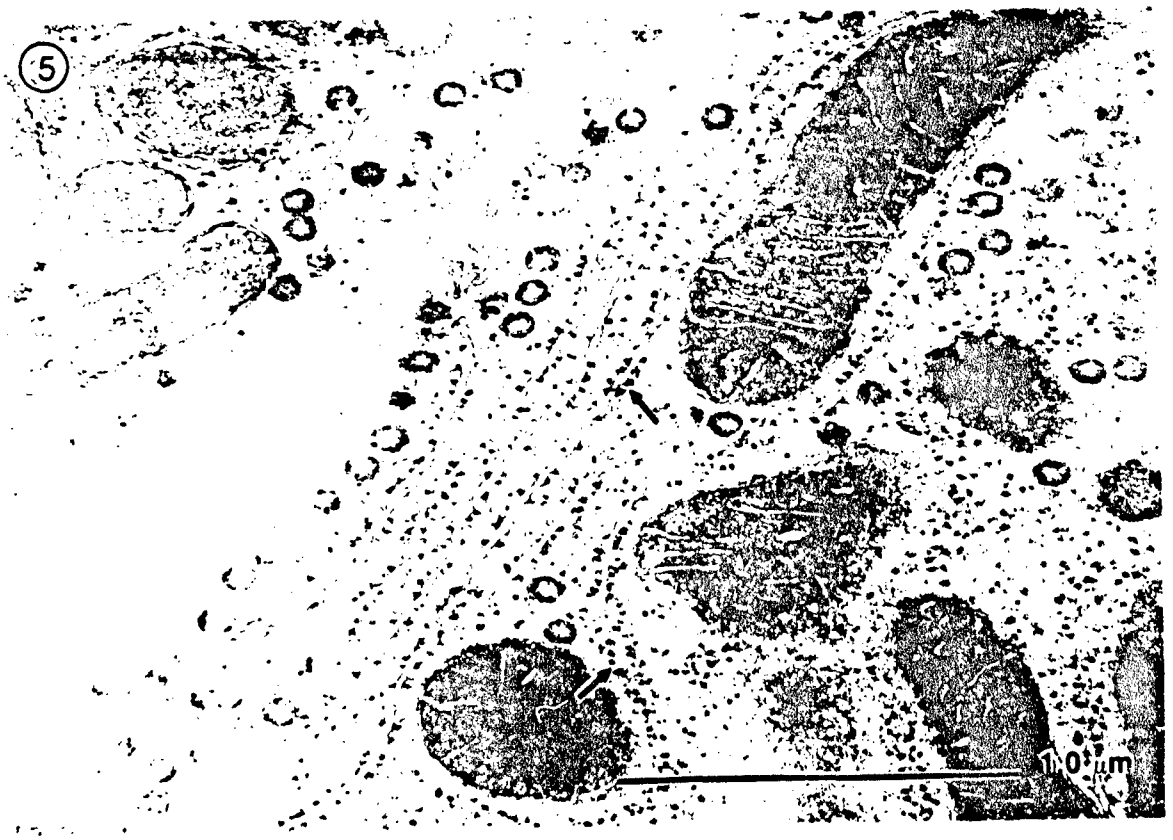
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